Replacing PAPS: *in vitro* phase II sulfation of steroids with the liver S9 fraction employing ATP and sodium sulfate

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Abstract:

In vitro technologies provide the capacity to study drug metabolism where *in vivo* studies are precluded due to ethical or financial constraints. The metabolites generated by *in vitro* studies can assist anti-doping laboratories to develop protocols for the detection of novel substances that would otherwise evade routine screening efforts. In addition, professional bodies such as the Association of Official Racing Chemists (AORC) currently permit the use of *in vitro* derived reference materials for confirmation purposes providing additional impetus for the development of cost effective *in vitro* metabolism platforms. In this work alternative conditions for *in vitro* phase II sulfation using human, equine or canine liver S9 fraction were developed, with adenosine triphosphate (ATP) and sodium sulfate in place of the expensive and unstable co-factor 3'-phosphoadenosine-5'-phosphosulfate (PAPS), and employed for the generation of six representative steroidal sulfates. Using these conditions, the equine *in vitro* phase II metabolism of the synthetic or so-called "designer" steroid furazadrol ([1',2']isoxazolo[4',5':2,3]-5 α -androstane-17 β -ol) was investigated, with ATP and Na₂SO₄ providing comparable metabolism to reactions using PAPS. The major *in vitro* metabolites of furazadrol matched those observed in a previously reported equine *in vivo* study. Finally, the equine *in vitro* phase II metabolism of the synthetic steroid superdrol (methasterone, 17 β -hydroxy-2 α ,17 α -dimethyl-5 α -androstane-3-one) was performed as a prediction of the *in vivo* metabolic profile.

Keywords:

sulfate ester, sulfation, *in vitro* metabolism, PAPS, steroid, anti-doping

1. Introduction:

The development of analytical methods to detect the use of illicit substances such as performance-enhancing Anabolic Androgenic Steroids (AAS) requires a detailed knowledge of drug metabolism.^{1,2,3} For many agents the parent compound is extensively metabolised and cannot be detected, so metabolites must be targeted as markers of drug administration. Traditionally, the metabolic profile of a steroid is established by *in vivo* studies that involve drug administration to one or more experimental subjects. Alternatively, *in vitro* technologies based on cultured liver cells or liver extracts provide methods that mitigate many of the ethical concerns regarding human or animal health and safety.^{4,5} These issues are of particular relevance in the case of synthetic or so-called “designer” steroids since they are often brought to market in a clandestine fashion, and the majority of these compounds do not have available data regarding their purity, safety, or efficacy.^{6,7} Given this, *in vitro* experiments would offer a safer approach to identifying metabolite markers.

The metabolism of AAS occurs in two complementary phases: phase I metabolism includes chemical modifications of the steroid skeleton such as reduction, oxidation, or hydroxylation, whereas phase II metabolism includes condensation reactions with other small molecules, termed conjugation. Steroid phase II metabolism usually involves conjugation with polar anionic sulfate or glucuronic acid groups to increase aqueous solubility for excretion, and in many cases these phase II conjugates make up the majority of metabolites detected.^{1,2,3} As a consequence, phase II metabolites can serve as markers for drug detection, and monitoring directly for these metabolites can offer advantages such as reduced sample preparation.⁸ Other advantages can also arise from the study of phase II metabolites. Monitoring of phase II sulfate conjugates can increase detection windows for some analytes,^{9,10,11,12,13,14} and has been used to distinguish between the endogenous or exogenously administered steroids.^{10,15,16,17} Further, the formation and subsequent decomposition of phase II sulfate conjugates has also been implicated in epimerisation of 17 α -alkyl-17 β -hydroxy steroids, leading to the formation of 17 β -alkyl-17 α -hydroxy compounds and other minor but significant metabolites.^{18,19,20} Given the importance of phase II metabolism, there is a need for *in vitro* systems that can faithfully replicate *in vivo* metabolism. This is particularly relevant in equine and canine sports, as current Association of Official Racing Chemist (AORC) criteria allow for the use of *in vitro*-derived reference materials in confirmatory analysis.²¹

In vitro technologies typically make use of enzymatic products derived from liver tissue, as the liver is the primary organ involved AAS metabolism. Homogenised liver can be centrifuged at 9,000 g for 20 min to isolate a supernatant commonly referred to as the S9 fraction.²² This fraction includes disrupted membranes of the endoplasmic reticulum (microsomes) and the soluble components of the cytosol. The S9 fraction can be fractionated further by ultracentrifugation at 100,000 g to isolate the microsomal fraction (pellet) from the cytosolic fraction (supernatant) and all three preparations are commercially available. From the perspective of AAS metabolism: microsomes are a concentrated source of cytochrome P450, flavin monooxygenase, and uridine 5'-diphospho-glucuronosyltransferase (UGT)

enzymes; liver cytosol contains aldehyde oxidase and sulfotransferase (SULT) enzymes; and S9 fraction contains all of these components. Cofactors must be added to these liver extracts for *in vitro* metabolism: glucuronylation by S9 fraction or microsomes with uridine 5'-diphoglucuronic acid (UDPGA), or sulfation (sometimes called sulfonation) by S9 fraction or cytosol with 3'-phosphoadenosine-5'-phosphosulfate (PAPS).⁴ However, the PAPS cofactor required for sulfation is prohibitively expensive (US\$1,645 for 25 mg of PAPS, Sigma-Aldrich²³) and chemically unstable (PAPS $t_{1/2}$ = 20 h at pH 8.0²⁴), and as a result *in vitro* studies are typically limited to phase I metabolism.^{5,25} There have been some reports detailing the study of phase II steroid metabolism using *in vitro* systems,^{26,27,28} but to date these have not been widely adopted by laboratories.

The limitations associated with PAPS can be overcome by *in situ* synthesis based on the physiological biosynthetic pathway (**Figure 1**).^{24,29–31} An *in vitro* approach for the preparation of sulfate metabolites has been described by Burkart *et al.*²⁴ and employs a series of six bacterial enzymes derived from *Rhizobium meliloti* and *Escherichia coli* for the *in situ* generation of PAPS. Using this approach, ATP-sulfurylase catalyses the sulfation of ATP to generate adenosine-5'-phosphosulfate (APS). This compound is subsequently phosphorylated by APS-kinase to generate PAPS and adenosine diphosphate (ADP). In animal cells, these two enzymes are expressed as a bifunctional protein molecule named PAPS synthase (PAPSS).³² Following PAPS synthesis, a sulfotransferase (SULT) can then catalyse the sulfation of a target molecule hydroxyl group. In addition to the sulfated metabolite, 3'-phosphoadenosine-5'-phosphate (PAP) is released and is subsequently dephosphorylated and re-phosphorylated in several steps to afford ATP. Overall this protocol generated a number of sulfate compounds in high yield, but may be difficult to implement due to the requirement for a number of bacterial enzymes, many of which are not readily available to laboratories.³³

Figure 1: Sulfotransferase promoted sulfation of an acceptor alcohol by PAPS together with the two-step biogenesis of PAPS from ATP and sodium sulfate promoted by ATP sulfurylase and APS kinase. In animal cells, these two enzymes are expressed as a bifunctional protein molecule PAPSS.

Sulfation of xenobiotics is known to occur in the liver, suggesting that the enzymes required for PAPS production would be present in liver preparations. The PAPSS isoform PAPSS2b localises in the cytoplasm and is therefore available for PAPS generation in the liver S9 fraction or cytosol.^{34,35,36,37} As a result, this study explored the possibility of employing ATP (US\$53 for 1000 mg, Sigma Aldrich³⁸) and sodium sulfate as inexpensive precursors for the generation of PAPS *in situ* for *in vitro* metabolism targeting sulfate metabolites. In particular, the study sought to optimise the experimental conditions and provide a metabolic platform capable of faithfully replicating *in vivo* sulfation. The optimised conditions have been used to generate equine *in vitro* phase II metabolic profiles of the synthetic anabolic steroids furazadrol (**Figure 2**, [1',2']isoxazolo[4',5':2,3]-5 α -androstan-17 β -ol, **F**)²⁵ and superdrol (methasterone, 17 β -hydroxy-2 α ,17 α -dimethyl-5 α -androstan-3-one, **S**),⁵ so demonstrating an economical approach for the study of phase II drug metabolism.

Figure 2: Chemical structures of furazadrol, **F** and superdrol, **S**.

2. Materials and Methods

2.1 Materials

Chemicals and solvents including lithium tri-*sec*-butylborohydride (L-Selectride®) solution in anhydrous tetrahydrofuran (THF), anhydrous *N,N*-dimethylformamide (DMF), magnesium chloride, sulfur-trioxide pyridine complex (SO₃.py), *tertiary*-butanol (*t*-BuOH), glucose-6-phosphate (G6P), NAD-dependant glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, adenosine triphosphate (ATP), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), nicotinamide adenine dinucleotide phosphate (NADP), uridine 5'-diphosphoglucuronic acid (UDPGA), and estrone (3-hydroxyestra-1,3,5(10)-trien-17-one, **E**) were purchased from Sigma-Aldrich (Castle Hill, Australia), and were used as supplied unless otherwise stated. Neutral ATP stock solutions were prepared at 0.1 M with aqueous sodium hydroxide solution. Nicotinamide adenine dinucleotide (NAD) was purchased from Amresco (Solon, USA). Formic acid was purchased from Ajax Chemicals (Auburn, Australia). 1,4-Dioxane (dioxane) was purchased from Merck (Darmstadt, Germany). Epiandrosterone (3 β -hydroxy-5 α -androstan-17-one, **EA**), androsterone (3 α -hydroxy-5 α -androstan-17-one, **A**), etiocholanolone (3 α -

hydroxy-5 β -androstan-17-one, **EC**), testosterone (17 β -hydroxyandrost-4-en-3-one, **T**) and nandrolone (17 β -hydroxyestra-4-en-3-one, **N**) were purchased from Steraloids (Newport RI, USA). Superdrol (**S**) was purchased from the National Measurement Institute (North Ryde, Australia). Epitestosterone (17 α -hydroxyandrost-4-en-3-one, **ET**) was prepared from **T** according to literature methods,³⁹ Epiandrosterone 3-sulfate (**EAS**), androsterone 3-sulfate (**AS**), etiocholanolone 3-sulfate (**ECS**), estrone 3-sulfate (**ES**), testosterone 3-sulfate (**TS**) and nandrolone 17-sulfate (**NS**) were prepared according to literature methods.⁴⁰ Pooled equine, canine, or human liver S9 fractions were purchased from Sekisui XenoTech (Kansas City, USA). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis WAX 6cc cartridges (PN 186004647), or Waters Sep-Pak C18 (3 cc, 500 mg) cartridges (PN WAT020805) as specified. *Escherichia coli* glucuronylsynthase, and α -D-glucuronyl fluoride were prepared according to literature methods.⁴¹ *Escherichia coli* NADP-dependant G6PDH was expressed with an N-terminal hexa-histidine tag purified according to previously described methods.⁴² The *E. coli* BL21 (DE3) with pETMCSIII-g6pdh was kindly provided by Professor David Ollis at the Research School of Chemistry, Australian National University.

2.1.2 Furazadrol reference materials

A range of previously synthesised furazadrol reference materials were employed to aid the identification of phase I and phase II metabolites.^{25,40} These were **F**, isofurazadrol ([1',2']isoxazolo[4',3':2,3]-5 α -androstan-17 β -ol, **IF**), epifurazadrol ([1',2']isoxazolo[4',5':2,3]-5 α -androstan-17 α -ol, **EF**), oxidised furazadrol ([1',2']isoxazolo[4',5':2,3]-5 α -androstan-17-one, **OF**), oxidised isofurazadrol ([1',2']isoxazolo[4',3':2,3]-5 α -androstan-17-one, **OIF**), furazadrol 17-sulfate (**FS**), isofurazadrol 17-sulfate (**IFS**), epifurazadrol 17-sulfate (**EFS**), furazadrol 17-glucuronide (**FG**), isofurazadrol 17-glucuronide (**IFG**), and epifurazadrol 17-glucuronide (**EFG**).

2.1.3 Superdrol reference materials

A range of superdrol reference materials were employed to aid the identification of phase I and phase II metabolites. Superdrol (**S**) was sourced commercially, however the remaining materials were prepared synthetically, including: 2 α ,17 α -dimethyl-5 α -androstan-3 α ,17 β -diol (**3 α -RS**), 2 α ,17 α -dimethyl-5 α -androstan-3 β ,17 β -diol (**3 β -RS**), 2 α ,17 α -dimethyl-5 α -androstan-3 α ,17 β -diol 3-sulfate (**3 α -RSS**), 2 α ,17 α -dimethyl-5 α -androstan-3 β ,17 β -diol 3-sulfate (**3 β -RSS**), and 2 α ,17 α -dimethyl-5 α -androstan-3 β ,17 β -diol 3-glucuronide (**3 β -RSG**). Experimental details, characterisation data, and copies of the ¹H NMR, selected ¹³C NMR, and +EI LRMS or -ESI LRMS spectra are provided in the Supporting Information.

2.2 Analytical methods

2.2.1 LC-MS assay for the optimisation of the *in vitro* sulfation reaction

The LC-MS analysis for the optimisation of the *in vitro* sulfation reaction was performed on an Agilent (Mulgrave Australia) 1260 UHPLC system coupled to an Agilent 6120 quadrupole mass spectrometer equipped with an Agilent Poroshell 120 EC-C18 column (2.1 mm x 30 mm, 2.7 μ m) incubated at 30 °C

and eluting with a gradient consisting of the following mobile phases, A: 10% methanol in aqueous ammonium acetate solution (10 mM), B: 90% methanol in aqueous ammonium acetate solution (10 mM), gradient: 0-5 min A-B (70:30 v/v) to B (100%), 5-6 min, B (100%), 6-9 min B (100%) to A-B (70:30 v/v), 6 min re-equilibration, flow rate 0.2 mL min⁻¹. Steroid sulfate conjugates were monitored for the anion, [M-H]⁻, using atmospheric pressure electrospray ionization (AP-ESI) and selected ion monitoring.⁴³

2.2.2 Furazadrol LC-MS analysis

The LC-MS analysis for furazadrol metabolites was performed as previously reported.²⁵ Positive mode liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry analysis was undertaken using a Thermo Fisher Scientific (Bremen, Germany) Ultimate 3000 HPLC coupled to an Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer equipped with a Waters SunFire C18 column (100 mm x 2.1 mm, 3.5 µm) eluting with a gradient consisting of the following mobile phases, A: 0.1% formic acid in water, B: 0.1% formic acid in methanol, gradient: 0-1 min A-B (95:5 v/v), 1-15 min A-B (95:5 v/v) to A-B (5:95 v/v), 15-19 min A-B (5:95 v/v), 5 min re-equilibration, flow rate 0.4 mL min⁻¹. Unconjugated steroids and steroid glucuronides were monitored for the proton adduct ([M+H]⁺) using HESI in positive full scan or targeted MS/MS mode at a resolution of 70,000 (FWHM). Negative mode LC-HRAM spectrometry analysis was undertaken using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a Phenomenex (Torrance CA, USA) Gemini C18 column (50 mm x 2 mm, 5 µm), eluting with a gradient consisting of the following mobile phases, A: aqueous ammonium acetate (0.01 M, pH 9.0), B: 0.1% acetic acid in acetonitrile, gradient: 0-2 min A-B (99:1 v/v), 2-8.5 min A-B (99:1 v/v) to A-B (20:80 v/v), 2.7 min re-equilibration, flow rate 0.5 mL min⁻¹. Steroid glucuronide and sulfate conjugates were monitored for the anion ([M-H]⁻) using HESI in negative full-scan or targeted MS/MS mode at a resolution of 70,000 (FWHM).

2.2.3 Superdrol LC-MS analysis

The LC-MS analysis for superdrol metabolites was performed using an Agilent 1290 Infinity II LC system coupled to an Agilent 6545 Q-ToF mass spectrometer equipped with a Phenomenex Gemini C18 column (50 mm x 2 mm, 5 µm) eluting with the gradient outlined for negative mode LC-MS analysis in section 2.2.2. Steroid glucuronide and sulfate conjugates were monitored for the anion ([M-H]⁻) using HESI in negative full-scan or targeted MS/MS mode.

2.3 *In vitro* phase II metabolism

2.3.1 Optimising sulfation reactions with liver S9 fraction

Reactions to test and optimise steroid sulfation were prepared with final concentrations of: 50 mM Tris.HCl for pH 7.4, 0.55 mg mL⁻¹ total protein from equine liver S9 fraction, 30 µM steroid, 5 mM MgCl₂, and various sulfation reagents. Nandrolone sulfate (NS) was used as an internal standard with a final concentration of 4 µM.

Sulfation reagents consisted of: 80 or 200 μ M PAPS, or ATP and Na_2SO_4 at a range of final concentrations. The ratio of ATP to sulfate was kept at 2:1, in keeping with the stoichiometry for PAPS synthesis (**Figure 1**). The final ATP concentration was tested from 2 to 32 mM with **EA** as a substrate to demonstrate 16 mM as optimal (Supporting Information **Figure S1**). The optimal MgCl_2 concentration was then determined to be 20 mM with 16 mM ATP and 8 mM sulfate (Supporting Information **Figure S2**).

Reactions were started by adding PAPS or sulfation reagent, with a final volume of 400 μ L, and incubated at 37 °C. Aliquots of 50 μ L were taken from reactions or standards at defined time points and quenched by mixing with 150 μ L of 66% (v/v) methanol in water. The quenched sample was centrifuged at 21000 *g* for 10 min, the supernatant transferred to a new vial and 20 μ L injected for LC-MS analysis (section 2.2.1). The **EAS** peaks were integrated with ChemStation software (Agilent), and the response ratio of **EAS** to **NS** (internal standard) was determined and compared to the response ratio for a 30 μ M **EAS** external standard.

2.3.2 Comparison among species and steroids

To test the general utility of ATP and Na_2SO_4 as a source of PAPS the amount of sulfation for six steroids at 30 μ M concentration: **A**, **EA**, **EC**, **E**, **T** and **ET** was tested. These were tested with three different liver S9 fractions: human, canine and equine. Reactions and analysis were performed as described above (section 2.3.1), except that the optimised concentrations were used for ATP (16 mM), sulfate (8 mM) and MgCl_2 (20 mM). Reactions were performed in triplicate and were incubated at 37 °C for 24 hours. Steroid sulfate peaks were integrated with ChemStation software, and the response ratio of steroid sulfate to **NS** (internal standard) was determined and compared to the response ratio for 30 μ M steroid sulfate external standard.

2.3.3 *In vitro* phase II metabolism of furazadrol using PAPS

In vitro phase II metabolism was performed by modification of a reported method.²⁵ A solution containing steroid (120 μ M, 250 μ L) in sodium phosphate buffer (100 mM, pH 7.4) and methanol (0.4%) was treated in order with the following solutions: aqueous magnesium chloride (1.0 M, 2.3 μ L), aqueous G6P (100 mM, 37.5 μ L), aqueous NAD (50 mM, 15 μ L), aqueous NADP (50 mM, 15 μ L), aqueous NAD-dependant G6PDH (40 units mL^{-1} , 12.5 μ L), aqueous NADP-dependant G6PDH (40 units mL^{-1} , 12.5 μ L), aqueous PAPS (1.6 mM, 22.5 μ L), additional aqueous magnesium chloride in association with PAPS (1.0 M, 2.5 μ L), aqueous UDPGA (610 μ M, 31 μ L), water (74 μ L), and equine liver S9 fraction (20 mg mL^{-1} , 25 μ L). The final solution (500 μ L) was then incubated in an open tube with agitation for 16 h at 37 °C. The reaction was quenched with acetonitrile (1 mL), centrifuged (2000 rpm, 5 min) to pellet solids, and the supernatant was decanted. Concentration of the supernatant under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol-water (5:95 v/v, 200 μ L) and transferred to a sealed vial for subsequent LC-MS analysis as per section 2.2. Control experiments excluding PAPS, UDPGA, both UDPGA and PAPS, all phase I co-factors, equine liver S9 fraction, and steroid respectively

were performed alongside the above reaction, with addition of water or buffer as required to maintain a constant final reaction volume and buffer concentration.

2.3.4 *In vitro* phase II metabolism of furazadrol and superdrol using ATP and Na₂SO₄

In vitro metabolism with ATP and Na₂SO₄ was performed as per section 2.3.3 with the following substitutions: the PAPS solution was replaced by addition of aqueous ATP (250 mM, 32 µL), aqueous Na₂SO₄ (100 mM, 40 µL), and additional aqueous magnesium chloride (1.0 M, 5.7 µL). The volume of water added was reduced to maintain a constant final reaction volume (500 µL). Control experiments analogous to those reported in section 2.3.3 were also performed alongside this reaction.

3 Results and Discussion

3.1 Steroid sulfation with liver S9 fraction, ATP and sodium sulfate

Sulfation of **EA** was examined to test the hypothesis that stable and readily available reagents, ATP and Na₂SO₄, could be added to liver S9 fraction leading to PAPS synthesis and SULT-catalysed sulfation. Sulfation was detected as **EAS** production and the concentrations of ATP, Na₂SO₄ and MgCl₂ were subsequently optimised (with ATP:Na₂SO₄ fixed at 2:1) as 16, 8, and 20 mM respectively (section 2.3.1). This optimised reaction was then compared with reactions using PAPS at 80 or 200 µM with 5 mM MgCl₂ (**Figure 3**). Equine liver S9 fraction, ATP and Na₂SO₄ displayed steady **EAS** production over the first six hours, with continued **EAS** production but declining rates up to 24 hours. Meanwhile, 80 µM PAPS afforded a similar initial rate over the first six hours but **EAS** production ceased thereafter. Productivity with PAPS was not greatly enhanced by increased concentration; 200 µM PAPS gave the lowest initial rate and only resulted in a slight increase in **EAS** production relative to 80 µM PAPS after 24 hours.

The three conditions of ATP and Na₂SO₄, 80 µM and 200 µM PAPS produced approximately 15, 6 and 7.5 µM **EAS** respectively after 24 h, with ATP and Na₂SO₄ displaying the greatest **EAS** production beyond six hours. However, even after 24 hours, this reaction only reached about 50% completion. In all cases excess sulfation reagents were insufficient to generate 30 µM **EAS** over 24 h and aside from limited time this could be due to competing substrates for SULTs present in the liver S9 fraction, PAPS instability or inactivation for SULTs over the course of the incubations. Alternatively, the PAPS precursor ATP may be subject to other biochemical transformations, thus limiting the PAPS synthesis and sulfation. Substrate and product inhibition also play a role in the reaction profiles. Substrate inhibition of human SULT2A1 has been observed for PAPS at a saturating concentration of dehydroepiandrosterone (DHEA), an unsaturated congener of **EA**.⁴⁴ Although this phenomenon is highly dependent on DHEA concentration, it could be the basis for the lower initial rates observed for 200 µM PAPS compared with 80 µM PAPS for **EAS** production.

Figure 3: A temporal analysis of **EAS** production *in vitro* using equine S9 fraction and different phase II cofactors. Reactions were performed as described in section 2.3.1 with 30 μM **EA** and either: 80 μM PAPS (red points), 200 μM PAPS (blue points), or 16 mM ATP, 8 mM Na₂SO₄ and 20 mM MgCl₂ (black points) as PAPS precursor. The error bars represent the standard deviation from three independent reactions.

The general applicability of ATP and Na₂SO₄ for *in vitro* PAPS synthesis and steroid sulfation was demonstrated for the six steroids and equine, human and canine S9 fractions (**Figure 4**). There were distinct differences among the three species for steroid sulfation. Both human and equine systems were capable of sulfating all six steroids with peak activity for **EAS** with equine and **ETS** with human S9 fractions. Production of **ES** was lowest for the S9 fractions from both these species. This result reflects the expression profile for human liver where the SULT responsible for androgen sulfation (SULT2A1) predominates and that for estradiol (SULT1E1) is only a minor component.^{36,37} In contrast, canine S9 fraction had greatest activity for **ES** and no detectable activity for **ECS**, **TS** or **ETS** production. This observation is in agreement with previous studies into canine metabolism where glucuronylation is the predominant form of phase II conjugation for steroids with sulfation making only a minor contribution.³ Furthermore, the major SULT expressed in canine liver, SULT1A1, has been expressed recombinantly and shown to sulfate estradiol, but not DHEA. In summary, ATP and Na₂SO₄ gave sulfate ester synthesis for a range of steroids using the liver S9 fractions derived from humans, horses and dogs and therefore serves as a suitable replacement for PAPS for *in vitro* metabolism studies. Attention then turned to the use of ATP and Na₂SO₄ as a replacement of PAPS for the *in vitro* metabolism of the synthetic steroids **F** and **S**.

Figure 4 Comparing sulfation of six different steroids (30 μM , indicated on the x-axis) with liver S9 fraction from three different species: human (dark grey), equine (light grey), and canine (black). Reactions were performed as described in section 2.3.2 with ATP and Na_2SO_4 as precursors for PAPS. The production of each steroid sulfate (μM) is presented on a logarithmic scale with the error bars indicating the standard deviation from three independent reactions.

3.2 Synthesis of steroid reference materials

The synthesis of the furazadrol reference materials used in this study has been described in a previous publication.²⁵ A number of phase I and II superdrol reference were prepared based on predicted patterns of equine metabolism² or the phase I metabolites tentatively assigned in a previous equine *in vitro* metabolism study.⁵ The reduction of **S** with sodium borohydride gave rise to a mixture of 3 α /3 β -hydroxy isomers **3 α -RS** and **3 β -RS**, favouring the 3 β -isomer **3 β -RS**.⁴⁵ On the other hand, reduction with L-Selectride®, which is a bulky reducing agent, exclusively gave rise to the 3 α -alcohol isomer **3 α -RS**. Performing these reactions on the milligram-scale proceeded smoothly but did not allow these isomers to be readily separated. As a result, the 3 β -isomer **3 β -RS** was prepared with a minor 3 α -impurity **3 α -RS**, which was carried through subsequent reaction steps. The pure 3 α -isomer **3 α -RS** was used to identify the presence of minor 3 α -isomer products generated during reactions of the 3 β -isomer **3 β -RS**.

Mono-sulfation of the 3 α /3 β ,17 β -diols to give **3 α -RSS**, and mixed **3 α -RSS**/**3 β -RSS** proceeded in high yield, using established methodology.⁴⁰ On the other hand, enzymatic glucuronylation afforded only the 3 β -isomer **3 β -RSG**.⁴¹ This was highlighted by the reaction of a mixture of 3 α /3 β -hydroxy isomers **3 α -RS**/**3 β -RS**, which afforded 2 α ,17 α -dimethyl-5 α -androstane-3 β ,17 β -diol 3-glucuronide **3 β -RSG** as the sole product after purification by SPE. Unreacted **3 α -RS** was isolated from the reaction mixture. The selectivity for the glucuronylation of 3 β -hydroxy steroids using this enzyme has been documented, and presumably reflects the substrate binding within the enzyme active site.⁴¹

3.3 *In vitro* phase II metabolism of furazadrol

In vitro studies typically involve only phase I metabolism, and phase II metabolism is not routinely explored, despite the fact that for *in vivo* studies, phase II conjugates are usually the major metabolites observed.^{1,2,3} The addition of phase II co-factors has the potential to modify phase I metabolism by selectively intercepting phase I intermediates, and as a result, the inclusion of *in vitro* phase II metabolism has the potential to generate a more detailed metabolic profile that can better match the *in vivo* profile.

To explore these issues, the phase I and phase II equine metabolism of the synthetic steroid **F** was investigated. This compound was chosen as it allowed comparison to a recently reported *in vivo* equine administration study, and a comparative phase I *in vitro* study (**F:IF** 9:1).²⁵ In this work, the metabolism reactions were conducted as previously reported with minor variations.²⁵ Together with the addition of phase II cofactors UDPGA and PAPS or ATP and sodium sulfate, the phase I cofactors NADH and NADPH were regenerated using G6P and appropriate G6PDH enzymes. Metabolism data were examined using mass filters for predicted metabolites formed from up to three metabolic transformations including oxidation, reduction and hydroxylation, with or without subsequent sulfation or glucuronylation. Metabolite peaks were identified where exact masses were observed within ± 10 ppm of the predicted mass, and by comparison with control experiments. Metabolites were also matched against synthesised reference materials where available. A comparison of the previously reported study²⁵ with the present study is outlined below (**Table 1**). A table of the observed metabolites, retention times, precursor ions and MS/MS fragments are reported in the Supporting Information, together with copies of extracted ion chromatograms and MS/MS spectra.

Table 1

The *in vitro* metabolism of furazadrol (**F:IF** 9:1) was conducted using both UDPGA with PAPS, and UDPGA with ATP and Na₂SO₄ (**Table 1**). Employing UDPGA and PAPS, major phase II metabolites were observed including **FS**, **IFS**, **FG**, **IFG**, and **EFG**, which were matched to reference materials. Additionally, **EFS** was not observed by comparison to the reference material, identical to observations from the *in vivo* study.²⁵ Minor unidentified phase II metabolites were observed including six hydroxylated furazadrol sulfate metabolites (**S1-S6**), one oxidised and hydroxylated furazadrol sulfate metabolite (**S7**), five hydroxylated furazadrol glucuronide metabolites (**G1-G4**, **G6**), and two oxidised and hydroxylated furazadrol glucuronide metabolites (**G9-G10**). Of these minor metabolites, **S2** was identified as a match with the previously reported *in vivo* study.²⁵ On the other hand, the *in vitro* phase II study employing UDPGA, ATP and Na₂SO₄, identified the same major phase II metabolites including **FS**, **IFS**, **FG**, **IFG**, and **EFG**. Again **EFS** was not observed. Minor unidentified phase II metabolites were observed including six hydroxylated furazadrol sulfate metabolites (**S1-S6**), one oxidised and hydroxylated furazadrol sulfate metabolite (**S8**), two hydroxylated furazadrol glucuronide metabolites (**G3**, **G5**), and three oxidised and hydroxylated furazadrol glucuronide metabolites (**G7**, **G8**, **G10**). Of

these minor metabolites, **S2**, and **S8** were identified as matches with the previously reported *in vivo* study.²⁵

A comparison of the phase II metabolites observed using both PAPS or ATP and Na₂SO₄ *in vitro* systems (**Table 1**) indicated that a majority of the metabolites were common. The major phase II metabolites **FS**, **IFS**, **FG**, **IFG**, and **EFG** were common to both systems and in both instances **EFS** was not observed. Thus the *in vitro* studies clearly indicate that **EF** serves as substrate for UGT promoted glucuronylation but not SULT promoted sulfation. This could arise due to different enzyme-substrate affinities or reaction rates for the competing conjugation processes. It is consistent with observed patterns of equine *in vivo* metabolism where 17 α -hydroxy metabolites are commonly found in the glucuronide, but not the sulfate, fraction.²

Although the major phase II metabolites were common to both *in vitro* systems, the minor phase II metabolites appeared to vary between systems, particularly for the glucuronides (**S1-S6**, **G3**, **G10** were common, **S7**, **G1**, **G2**, **G4**, **G6**, **G9** PAPS only, **S8**, **G5**, **G7**, **G8** ATP and Na₂SO₄ only). The differences in the glucuronide profile may be attributed to the different sulfation reagents used in these systems. In the experiment utilising 80 μ M PAPS, the co-factors are added at the start of the metabolism reaction, and PAPS is then consumed or decays in solution over time (**Figure 3**). This results in a burst of phase II sulfation towards the beginning of the reaction, which ceases relatively quickly. This is in contrast to the experiments utilising ATP and Na₂SO₄ in which a relatively steady rate of sulfation was observed. Of particular interest were the minor metabolites **S2** and **S8**, which were both observed in the previous *in vivo* study.²⁵ The *in vitro* phase II study employing PAPS identified **S2** only, while the study employing ATP and Na₂SO₄ identified both **S2** and **S8** as minor metabolites, suggesting that *in vitro* platforms employing ATP and Na₂SO₄ could offer some advantages for generating some phase II metabolites.

A comparison of the phase I metabolites observed using both PAPS, and ATP/Na₂SO₄ *in vitro* systems indicated that a majority of the metabolites were common (**EF**, **OF**, **OIF**, **M2-M5**, **M8**, **M9**, **M12**, **M13**, **M15**, **M16**), with a few exceptions (**M1**, **M10**, **M14** PAPS only; **M6**, **M7**, **M11** ATP and Na₂SO₄ only). The number of phase I metabolites observed did not appear to change significantly in control experiments where phase II co-factors were excluded, suggesting that phase II metabolism was not fully intercepting the phase I metabolites. The extent of phase I metabolism in the present study appeared to be somewhat greater than reported previously.²⁵ The present study used a combination of NADH and NADPH co-factors with G6P and both *E. coli* and *L. mesenteroides* G6PDH enzymes for regeneration of both. In contrast, the previous study used NADH G6P and *L. mesenteroides* G6PDH. Although liver extracts enable NADH to reduce NADP⁺ and *vice versa*, additional NADPH for cytochrome P450 oxidation could explain the modest increase in the number of hydroxylated metabolites identified in the present study. For both systems, metabolites, **EF**, **OF**, **OIF**, **M2-M4**, and **M8** were identified as matches with the phase I study reported previously.²⁵ Although no unconjugated metabolites were identified in the previous *in vivo*

study,²⁵ the hydroxylated furazadrol **M4** was observed to match a phase I metabolite generated after *Pseudomonas aeruginosa* arylsulfatase hydrolysis of the *in vivo* samples.^{25 43}

It appears that the substitution of PAPS with ATP and Na₂SO₄ is capable of generating comparable *in vitro* metabolic profiles, and can serve as an economical alternative for the generation of *in vitro* phase II sulfate conjugates. The major metabolites **FS**, **IFS**, **FG**, **IFG**, and **EFG**, which include the most useful markers for anti-doping screening²⁵ were all detected at higher levels (response ratios 10-10³ times) than the minor phase II metabolites in these studies. On the other hand, the minor *in vivo* metabolites **S2**, and **S8** identified in the *in vitro* study would likely be difficult to distinguish from the other minor phase II metabolites observed. The long-term metabolites most suitable for use as screening markers are not always the most abundant,² and so may be difficult to identify solely from *in vitro* data. Although it could be argued from these results that the reported conditions are capable of generating an adequate representation of the major *in vivo* metabolites, further work refining these conditions to better match the complete *in vivo* profile solely from *in vitro* results is likely to be beneficial. For equine and canine sports, AORC criteria allow for the use of *in vitro*-derived materials as reference materials in confirmatory analysis.²¹ Improved methods to mimic the *in vivo* profile using *in vitro* techniques will generate new reference materials and allow additional instances of AAS misuse to be detected and confirmed.

3.4 *In vitro* phase II metabolism of superdrol

Given the success of investigations into the metabolism of **F**, attention turned to the equine *in vitro* phase II metabolism of **S**. The equine *in vivo* metabolism of **S** has not been previously reported and in the absence of this information this study serves as a prediction of the likely metabolic profile of this steroidal agent. Previous reports of the equine *in vitro* phase I metabolism of **S** provided some comparison with the present study.⁵ Several human *in vivo* and *in vitro* studies of **S** metabolism have also been reported.^{46,47,48,49} Metabolism of **S** with UDPGA, ATP, Na₂SO₄, and equine liver S9 fraction afforded a range of phase II sulfate and glucuronide metabolites which are summarised in **Figure 5**. A table of the observed metabolites, retention times, precursor ions and MS/MS fragments are reported in the Supporting Information, together with copies of extracted ion chromatograms and MS/MS spectra.

Figure 5: Proposed *in vitro* equine metabolism of superdrol (S); ^AMatched to reference material; ^BUnconfirmed regio- and stereo-chemistry. H, hydroxylation; R, reduction; G, glucuronylation; S, sulfation. Dotted boxes indicate presumed intermediates that were not observed in this LC-MS study.

Major phase II metabolites were observed including **3 β -RSS** which was matched to the available reference material, and a reduced superdrol glucuronide metabolite **G12**.^{48,49} Although no reference material was available, **G12** was tentatively assigned as 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-glucuronide (or its 17-glucuronide regioisomer), as this metabolite did not match the reference material for **3 β -RSG** but exhibited similar mass spectrometry behaviour including the proton loss species m/z 495 ([M-H]⁻) and the glucuronide derived fragments m/z 113 (C₅H₅O₃⁻), m/z 85 (C₄H₅O₂⁻) and m/z 75 (C₂H₃O₃⁻).⁵⁰ A metabolite corresponding to 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-glucuronide (or its 17-glucuronide regioisomer) has also been indirectly observed in human *in vivo* and *in vitro* studies of **S** metabolism.^{48,49} Despite this, some caution is required as the formation of stereoisomeric

17 β -methyl-17 α -hydroxy metabolites through formation and hydrolysis of the tertiary sulfate conjugate provides alternative metabolic pathways.^{18,19,20} Minor phase II metabolites were observed including two hydroxylated superdrol sulfate metabolites (**S9**, **S10**), a reduced and hydroxylated superdrol sulfate metabolite (**S11**), superdrol glucuronide (**G11**), **3 β -RSG**, and two reduced and hydroxylated superdrol glucuronide metabolites (**G13**, **G14**). Although the majority of minor phase II metabolites remain unidentified in this study, the minor metabolite **3 β -RSG** was confirmed, and **3 α -RSS** was not observed, by comparison to the corresponding reference materials.

The formation of the 3 β -hydroxy metabolites is known to be favoured in horses,² and this is followed by sulfation to give major **3 β -RSS** and glucuronylation to give minor **3 β -RSG**. Alternatively, the tentative assignment above suggests that formation of the 3 α -hydroxy metabolite **3 α -RS** is followed by glucuronylation to give major **G12**^{48,49} but not sulfation to **3 α -RSS**, in a pattern similar to that observed in humans.¹ Although no unconjugated phase I metabolites were identified directly in this LC-MS study, the phase I metabolism of **S** is known to afford a number of steroid diol, and triol metabolites which ionise poorly under +ESI conditions.⁵ Instead, these metabolites were identified indirectly through analysis of their intact phase II conjugates. Future studies of the *in vivo* metabolism of **S** are likely to benefit from access to a wider variety of reference materials and the application of both GC-MS and LC-MS for sample analysis. Overall, the phase I metabolism of **S** appears to be quite simple, and the major phase II conjugates observed correlate well with the major phase I metabolites previously reported for the equine *in vitro* studies.⁵ The reduced superdrol isomers **3 β -RS**, and **3 α -RS** appear to be the most abundant *in vitro* metabolites observed, and would likely be among the major metabolites observed *in vivo*. Additionally, these compounds are also easily prepared from the parent compound, and so should be available as reference materials to assist in confirmation. As such, it is recommended that anti-doping laboratories monitor for **3 β -RSS**, and 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-glucuronide (tentatively identified as **G12**), or their phase I counterparts **3 β -RS**, and **3 α -RS**, until such time as a comparative *in vivo* study can be undertaken.

4 Conclusion

In this work alternative conditions for *in vitro* phase II sulfation using human, equine or canine liver S9 fraction were developed and employed for the generation of six representative steroidal sulfates, with ATP and Na₂SO₄ in place of the expensive and unstable co-factor PAPS. Using these conditions, the equine *in vitro* phase II metabolism of the synthetic steroid **F** was investigated, with ATP and Na₂SO₄ found to offer comparable metabolism to reactions using PAPS. The major *in vitro* metabolites of **F** also correlated closely with the metabolites observed in a previously reported equine *in vivo* study.²⁵ Although these *in vitro* methods do not fully replicate the *in vivo* metabolic profile, it is expected that future work will refine these methods to more accurately predict the metabolism of new compounds from *in vitro* studies. Finally, the equine *in vitro* phase II metabolism of the synthetic steroid **S** was also performed that serves as a prediction of the *in vivo* metabolic profile. Notably the equine *in vitro* phase II metabolites observed in this study correspond well with previously reported equine *in vitro* phase I studies and so are recommended as suitable screening markers until *in vivo* studies can be undertaken.

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Table 1: Comparison of *in vivo* and *in vitro* (UDPGA and PAPS, or UDPGA, ATP and Na₂SO₄) metabolism of furazadrol (**F:IF** 9:1); ^ASee ref ²⁵; ^BN/D not detected.

Metabolic transformation	<i>In vivo</i> (previous study) ^A	Phase II <i>in vitro</i> (UDPGA and PAPS)	Phase II <i>in vitro</i> (UDPGA, ATP, and Na ₂ SO ₄)	Phase I <i>in vitro</i> (previous study) ^A	Phase I <i>in vitro</i> (UDPGA and PAPS)	Phase I <i>in vitro</i> (UDPGA, ATP, and Na ₂ SO ₄)
furazadrol (major)	FS, IFS, FG, IFG, EFG	FS, IFS, FG, IFG, EFG	FS, IFS, FG, IFG, EFG	EF	EF	EF
oxidised furazadrol (major)	N/D ^B	N/D ^B	N/D ^B	OF, OIF	OF, OIF	OF, OIF
hydroxylated furazadrol (minor)	sulfate (x 1)	sulfate (x 6) S1-S6 glucuronide (x 5) G1-G4, G6	sulfate (x 6) S1-S6 glucuronide (x 2) G3, G5	(x 8)	(x 7) M1-M5, M8, M9	(x 8) M2-M9
oxidised and hydroxylated furazadrol (minor)	sulfate (x 2) glucuronide (x 2)	sulfate (x 1) S7 glucuronide (x 2) G9, G10	sulfate (x 1) S8 glucuronide (x 3) G7, G8, G10	(x 1)	(x 2) M10, M12	(x 2) M11, M12
dihydroxylated furazadrol (minor)	N/D ^B	N/D ^B	N/D ^B	(x 2)	(x 4) M13-M16	(x 3) M13, M15, M16